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PURIFICATION OF *CLOSTRIDIUM BOTULINUM* TYPE A TOXIN

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SUMMARY

The neurotoxin of *Clostridium botulinum* Type A has been isolated and purified from a liquid culture. The toxin is homogenuous by anion and cation exchange chromatography, gel filtration, isoelectric focusing and Ouchterlony gel diffusion technique. The specific toxicity of the purified toxin is $10 \cdot 10^7$ minimum lethal doses/1.0 $A_{278\ nm}$, the molecular weight is 150 000 by gel filtration method and the isoelectric point is pH 6.1. This preparation could not be distinguished from the α fraction isolated from the crystalline toxin. Results presented here failed to confirm the claim of GERWING *et al.*[5] that a toxin of 12 200 mol. wt. can be isolated from the liquid culture of this organism.

INTRODUCTION

There are five methods for the purification of the toxic protein of *Clostridium botulinum* Type A (refs. 1–5) yet none of the methods yields a homogenous preparation. The crystalline toxin of mol. wt. 900 000 obtained by four of these methods[1–4] has been found to be a mixture of the toxin (the α fraction) and another protein with hemagglutinating activity[6–9]. Isolation of a homogenous toxin preparation of mol. wt. 12 200 by GERWING *et al.*[5] has been disputed[10–12]. Since there is no method available for isolation of the pure toxin from the bacterial culture of *C. botulinum* Type A a procedure to obtain a homogenous toxin preparation has been developed. In this paper (i) such a method is described and (ii) the toxin purified by this method is compared with the toxin (the α fraction) isolated from the crystalline preparation.

RESULTS

The organisms from a frozen culture inoculum were grown in a dialysis sac immersed in the medium[13]. The culture produced maximum toxicity ($4 \cdot 10^6$–$6 \cdot 10^6$ M.L.D.*/ml) after 96 h of incubation at 37°. Toxicity was assayed according to BOROFF AND FLECK[14]. The culture was freed from organisms by centrifugation at 14 600

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* Number of minimal lethal doses.

$\times g$ for 40 min followed by filtration through a 1.2-μ Millipore filter. Solid $(NH_4)_2SO_4$ was added slowly (31.3 g/100 ml) with gentle stirring to the culture filtrate at room temperature. After 48 h at 4° the precipitate was collected by centrifugation at 27 000 $\times g$ for 20 min. The pellet was suspended in 0.067 M citrate–phosphate buffer (pH 5.6) (ref. 8) in a volume equal to 2% of the culture filtrate volume. This material was dialyzed against 15 vol. of the pH 5.6 buffer at 4° for 2 h with two changes of the buffer. The insoluble material that appeared during dialysis was removed by centrifugation at 12 000 $\times g$ for 20 min. The toxic supernatant in 4.0-ml portions was applied to DEAE-cellulose columns (1.0 cm $\times$ 30 cm) equilibrated with the pH 5.6 buffer. The column was eluted with the same buffer. A large sharp peak emerged without being held on the column. The front edge of the peak was water-clear but thereafter the fractions had a faint yellowish color. Fractions across the peak with $A_{278\ nm}$ of 0.1, and above were pooled. The pooled material is referred to as pH 5.6 toxin. Occasionally, the crude toxin from different batches of culture fluid produced a shoulder on the trailing edge of the peak. Whenever this happenend, the fractions under the shoulder were discarded. The reason for the variability of the chromatographic profile arising from different bacterial culture filtrates is not known.

The pH 5.6 toxin was applied in 10.0-ml portions to 2.2 cm $\times$ 42 cm Sephadex G-100 columns equilibrated and eluted with the pH 5.6 buffer. The elution profile showed a major peak at the void volume followed by two smaller peaks (Fig. 1). Most of the toxic activity applied to the column was found in the first peak. Fractions across this peak (referred as G-100 toxic peak) with $A_{278\ nm}$ of 0.045, and above were

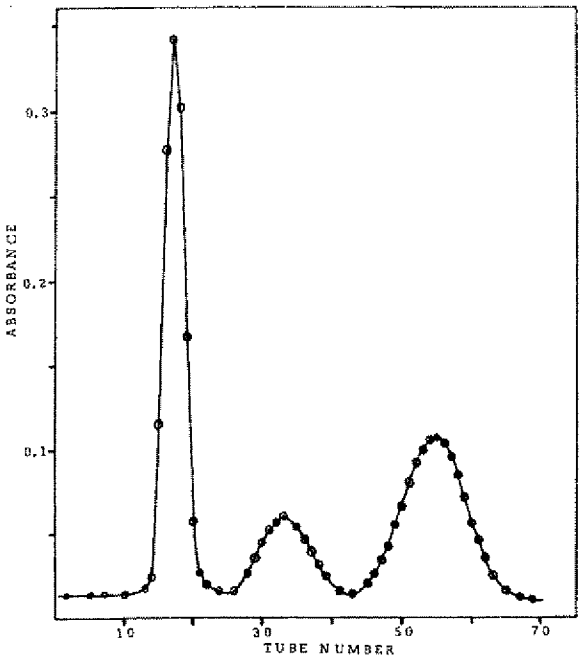

Fig. 1. Gel filtration of 10.0 ml of pH 5.6 toxin of 0.95 $A_{278\ nm}$, on a 2.2 cm $\times$ 42 cm Sephadex G-100 column equilibrated and eluted with 0.067 M citrate–phosphate buffer (pH 5.6). Fractions of 3.0 ml were collected at a flow rate of 30 ml/h.

pooled. Solid $(NH_4)_2SO_4$ was added slowly with gentle stirring to the pooled G-100 toxic peak (39 g/100 ml) at room temperature and was left for 48 h at 4°. The precipitated toxin was collected by centrifugation at 39 000 × *g* for 20 min and suspended in 0.15 M Tris–HCl buffer pH 8.0 (ref. 8) in a volume equal to 2% of the total G-100 toxic peak volume. Most of the pellet dissolved within 1 h at room temperature. The suspension was dialyzed against 10 vol. of the pH 8.0 buffer for 4 h with an hourly change of the dialyzing buffer. The insoluble material in the dialysis sac was removed by centrifugation (International clinical centrifuge, at half the maximum speed for 10 min).

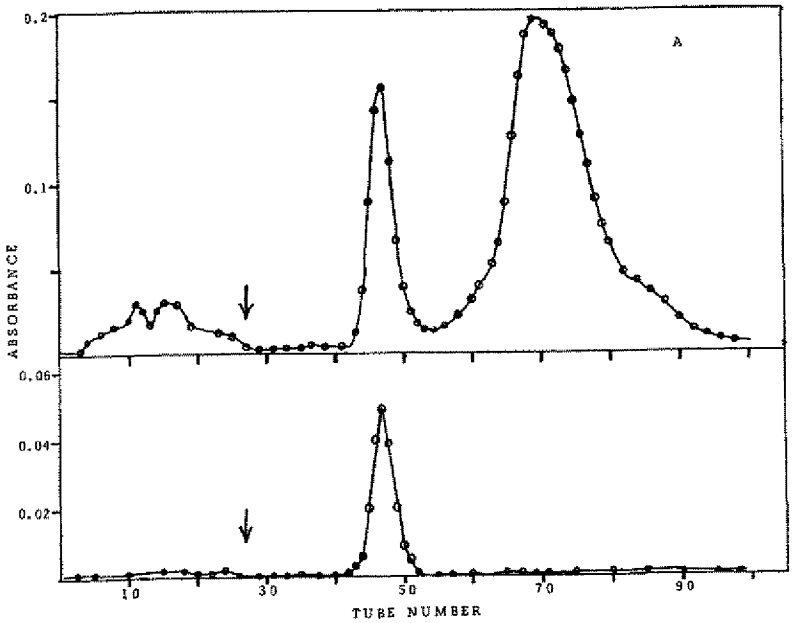

Fig. 2. A. Chromatography of 8.2 ml of supernatant of 2.47 $A_{278\ nm}$, on a 1.0 cm × 30 cm DEAE-Sephadex A-50 column equilibrated with 0.15 M Tris–HCl buffer (pH 8.0). A linear gradient elution (130 ml of the equilibrating buffer *plus* 130 ml of equilibrating buffer containing 0.3 M NaCl) was commenced at Tube No. 27 marked with an arrow. Fractions of 3.2 ml were collected at a flow rate of approx. 20 ml/h. Specific toxicity (M.L.D./1.0 $A_{278\ nm}$) of the eluate in Tube No. 44, 46, 47, 49, and 50 were $11.08 \cdot 10^7$, $11.15 \cdot 10^7$, $9.0 \cdot 10^7$, $10.6 \cdot 10^7$, and $9.0 \cdot 10^7$, respectively The mean specific toxicity across the peak was $10.14 \cdot 10^7$ M.L.D./1.0 *A*. B. Rechromatography of 10 ml of pooled eluate of *A* 0.073 from the second peak in A. Conditions of chromatography were the same as above. Linear gradient was started at arrow.

The supernatant was applied to a 1.0 cm × 30 cm DEAE-Sephadex A-50 column equilibrated with the pH 8.0 buffer. A small peak emerged on washing the column with the equilibrating buffer (Fig. 2A). Subsequently, a linear gradient of increasing Cl^- concentration eluted two clearly separated peaks. The material in the first peak was neither toxic nor hemagglutinating. The second peak was highly toxic and free of hemagglutinating activity. The specific toxicity of the eluate in the second peak was uniform across the peak and had a mean value of $10.1 \cdot 10^7$ M.L.D./1.0 $A_{278\ nm}$. The third peak contained strong hemagglutinating activity and mild toxicity. The material in the second peak is the neurotoxin of *C. botulinum* Type A. The overall

TABLE I

SUMMARY OF PURIFICATION

The data in the first two columns show the range of specific toxicity and recovery of toxic activity in each step of purification in six separate preparations. The mean values of recovery of toxicity with respect to the bacterial culture filtrate are shown in the last column.

Purification steps	*Specific toxicity**	*Recovery of toxic activity (%) from*	
		Preceding step	*Starting material*
1. Culture filtrate	$6.3 \cdot 10^4$–$7.3 \cdot 10^4$	100	100
2. Crude toxin $(NH_4)_2SO_4$ precipitate dissolved in pH 5.6 buffer	$1.9 \cdot 10^6$–$2.4 \cdot 10^6$	80–96	88
3. Supernatant of dialyzed crude toxin	$2.1 \cdot 10^6$–$2.5 \cdot 10^6$	47–72	58
4. pH 5.6 toxin (peak from DEAE-cellulose column)	$11.8 \cdot 10^6$–$16.8 \cdot 10^6$	59–72	38
5. Sephadex G-100 toxic peak (first peak from Sephadex G-100 column)	$20.4 \cdot 10^6$–$33 \cdot 10^6$	102–110	42
6. Pure toxin (toxic peak from DEAE-Sephadex A-50 column)	$9 \cdot 10^7$–$11.5 \cdot 10^7$	30–33	14

* Number of minimum lethal doses (M.L.D.)/1.0 $A_{278\ nm}$ in 1.0-cm light path.

purification achieved was about 1500-fold (Table I). The toxin thus obtained was tested for homogenity and other properties.

Ten ml of the purified toxin was dialyzed against 200 ml of pH 8.0 buffer for 4 h at 4° with hourly change of the buffer. This removed NaCl and the dialysate was recycled through a DEAE-Sephadex A-50 column under conditions identical to those described above. Only one peak emerged (Fig. 2B). The apex of this peak was at the same position after commencement of the gradient as that of the second peak in the previous run (Fig. 2A).

Chromatography of a portion of the purified toxin on a IRC-50 (XE-64) column equilibrated at pH 5.55, as described before[15], eluted only one peak (Fig. 3). Aliquots taken from various fractions under the peak proved toxic. Specific toxicity

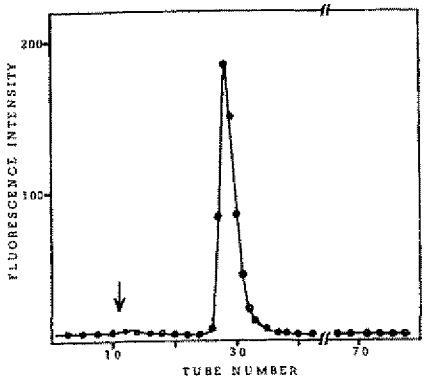

Fig. 3. Chromatography of the purified toxin on a 0.9 cm x 14 cm column of Amberlite IRC-50 (XE-64) equilibrated with 0.15 M citrate buffer (pH 5.55). Linear gradient elution (100 ml of the equilibrating buffer *plus* 100 ml of 0.5 M trisodium citrate) was commenced at Tube No. 11 marked with an arrow. Fractions of 2.8 ml were collected at a flow rate of 18–20 ml/h.

of these fractions could not be measured in terms of M.L.D. $A_{278\ nm}$ unit because of low absorbance readings.

One ml of the pure toxin was eluted through a 1.5 cm × 60 cm Sephadex G-200 column. The column was equilibrated and eluted with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1 M KCl (ref. 8). The toxin emerged as a single peak with no visible inflection points on the leading or the trailing edge of the peak (Fig. 4). Horse heart cytochrome *c*, bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle aldolase, catalase, β-galactosidase and blue dextran were also eluted through the same column under the same conditions. The elution volume of these substances plotted against the logarithm of their molecular weight yielded a straight line (not shown here). This curve was used to estimate the molecular weight of the toxin[16]. The elution volume of the toxin and of aldolase were the same. Thus the molecular weight of the toxin is believed to be about 150 000 since the molecular weight of aldolase is 149 000 (ref. 17).

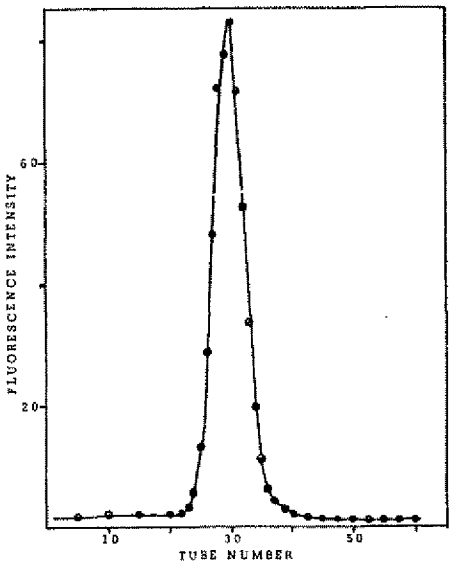

Fig. 4. Gel filtration of 1.0 ml of purified toxin of 0.38 *A* on a 1.5 cm × 60 cm column of Sephadex G.200. The column was equilibrated and eluted with 0.05 M Tris–HCl buffer (pH 8.0) containing 0.1 M KCl. Fractions of 1.92 ml were collected at a flow rate of 15 ml/h. The elution volume of blue dextran, toxin and rabbit muscle aldolase were 38.4, 57.6 and 57.6 ml, respectively.

Three different preparations of the purified toxin were analyzed by isoelectric focusing. In each case one protein peak could be observed in the eluted material. In two experiments when the anode was at the bottom of the column, the isoelectric pH of the toxin was found to be 6.1 and 6.23. In a third experiment with the anode at the top of the column the isoelectric point was pH 6.08 and in good agreement with the value 6.1. The isoelectric pH of the toxin was therefore judged to be 6.1. Fig. 5 shows the profile of fluorescence intensity of the eluted material, the pH gradient and toxicity of the protein peak fractions.

The Type A toxin purified by the method described above, the α fraction isolat-

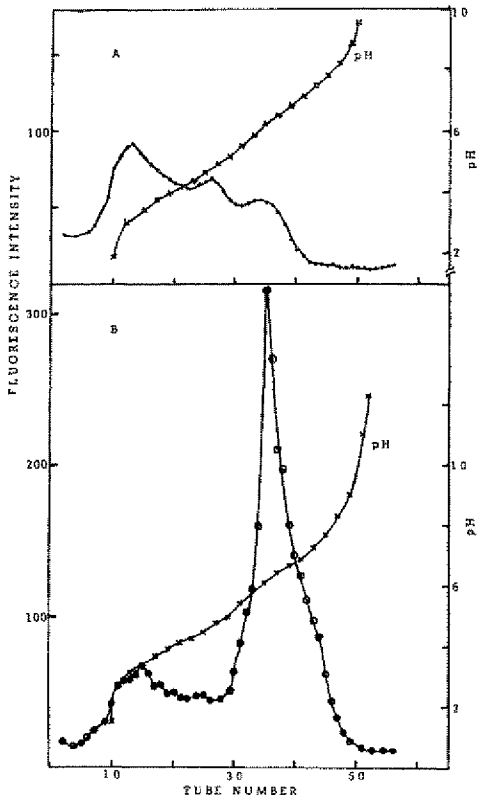

Fig. 5. pH and fluorescence intensity profile of eluates from an isoelectric focusing column (anode at the bottom of the column). A. Result of a blank run with no protein sample applied. B. Result with 2.0 ml of purified toxin of 0.38 *A* applied on the column. Toxicity of Fractions No. 32, 35 and 38 were $1.9 \cdot 10^5$, $3.2 \cdot 10^6$ and $1.9 \cdot 10^6$ M.L.D./ml, respectively. Ampholyte of pH range 3–10 (LKB Batch No. 22) in 1% (w/v) concentration was used in a 110-ml column. During 68 h of operation at 650 V the temperature of water circulating through the column jacket was maintained at 10°. The contents of the column were eluted in 1.9-ml fractions. Presence of protein in these fractions was established by measuring their fluorescent intensity. A few fractions containing protein were dialyzed for 10 h against 0.9% NaCl solution and tested for toxicity.

Fig. 6. Ouchterlony gel diffusion assay of the purified toxin and the α fraction. Central well contained antiserum to the purified toxin. Lateral wells No. 1 and 3 contained the α fraction, well No. 2 contained the purified toxin and well No. 4 contained Type B botulinum toxin.

ed from the crystalline toxin, and Type B toxin purified by the method described elsewhere[13] were placed in the lateral wells. The central well was filled with rabbit antiserum against the Type A toxin. Each antigen except Type B toxin produced one precipitation band. These bands coalesced at their ends indicating their serological identity (Fig. 6). As expected, Type B toxin did not react with the antiserum thereby demonstrating the serological nonidentity between Type A and Type B toxins.

The toxin purified by the present method and the α fraction isolated from the crystalline toxin were mixed and cochromatographed on DEAE-Sephadex A-50, Amberlite IRC-50 (XE-64) and Sephadex G-200 columns as described above. In each case the elution profile showed one peak a result similar to that found when only the toxin was chromatographed (see Figs. 2B, 3, 4).

DISCUSSION

The toxin isolated and purified by the present method is free of hemagglutinin and is homogenuous so far as can be determined by anion and cation exchange chromatography, gel filtration, Ouchterlony gel diffusion test and by isoelectric focusing technique. This toxin preparation cannot be distinguished from the fraction isolated from crystalline toxin. The specific toxicity of the purified toxin is $10 \cdot 10^7$ M.L.D./1.0 $A_{278\ nm}$ and is similar to that of the α fraction $9 \cdot 10^7$ M.L.D./1.0 $A_{278\ nm}$ (ref. 13). The molecular weight of the purified toxin determined by gel filtration method is 150 000 and is same as that of the α fraction[8]. Recovery of 14% of the total activity present in culture filtrate by the method presented compares favourly with the 5–20% and 17% recovery in the form of crystalline toxin[2,4]. It is significant however, that whereas crystalline toxin is about 80% impure the toxin purified by this method is free of detectable impurity. This establishes the validity of the purification procedure described here.

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REFERENCES

1 C. LAMANNA, O. E. MCELROY AND H. W. EKLUND, *Science*, 103 (1946) 613.
2 C. LAMANNA, H. W. EKLUND AND O. E. MCELROY, *J. Bacteriol.*, 52 (1946) 1.
3 A. ABRAMS, G. KEGELES AND G. A. HOTTLE, *J. Biol. Chem.*, 164 (1946) 63.
4 J. T. DUFF, G. G. WRIGHT, J. KLERER, D. E. MOORE AND R. H. BIBLER, *J. Bacteriol.*, 73 (1957) 42.
5 J. GERWING, C. E. DOLMAN AND H. S. BAINS, *J. Bacteriol.*, 89 (1965) 1383.
6 B. R. DASGUPTA, D. A. BOROFF AND E. ROTHESTEIN, *Biochem. Biophys. Res. Commun.*, 22 (1966) 750.
7 B. R. DASGUPTA AND D. A. BOROFF, *Biochim. Biophys. Acta*, 147 (1967) 603.
8 B. R. DASGUPTA AND D. A. BOROFF, *J. Biol. Chem.*, 243 (1968) 1065.
9 C. LAMANNA AND C. J. CARR, *Clin. Pharmacol. Therap.*, 8 (1967) 286.
10 E. J. SCHANTZ, *Japan J. Microbiol.*, 11 (1967) 380.
11 A. H. W. HAUSCHILD AND R. HILSHEIMER, *Can. J. Microbiol.*, 15 (1969) 1129.
12 J. N. KNOX, W. P. BROWN AND L. SPERO, *Infection and Immunity*, 1 (1970) 205.
13 B. R. DASGUPTA, D. A. BOROFF AND K. CHEONG, *Biochem. Biophys. Res. Commun.*, 32 (1968) 1057.
14 D. A. BOROFF AND U. FLECK, *J. Bacteriol.*, 92 (1966) 1580.
15 B. R. DASGUPTA, D. A. BOROFF AND K. CHEONG, *Biochim. Biophys. Acta*, 168 (1968) 522.
16 P. ANDREWS, *Biochem. J.*, 96 (1965) 595.
17 W. C. DEAL, W. J. RUTTER AND K. E. VANHOLDE, *Biochemistry*, 2 (1963) 246.